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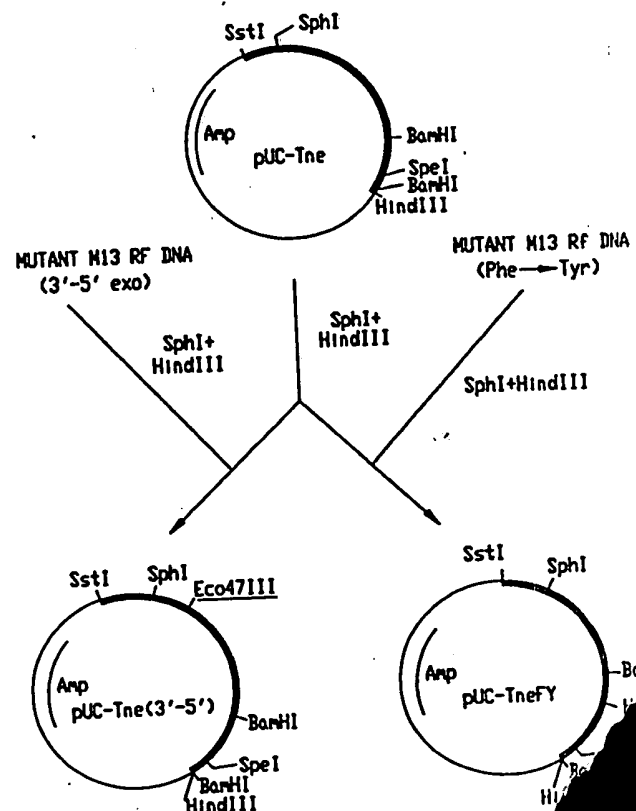
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(54) Title: CLONED DNA POLYMERASES FROM THERMOTOGA NEAPOLITANA AND MUTANTS THEREOF

(57) Abstract

The invention relates to a substantially pure thermostable DNA polymerase from *Thermotoga neapolitana* (Tne) and mutants thereof. The Tne DNA polymerase has a molecular weight of about 100 kilodaltons and is more thermostable than Taq DNA polymerase. The mutant Tne DNA polymerase has at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3' → 5' exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or eliminates 5' → 3' exonuclease activity of said DNA polymerase; (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against dideoxynucleotides. The present invention also relates to the cloning and expression of the wild type or mutant Tne DNA polymerase in *E. coli*, to DNA molecules containing the cloned gene, and to host cells which express said genes. The Tne DNA polymerase of the invention may be used in well-known DNA sequencing and amplification reactions.



Background Information

DNA polymerases synthesize the formation of DNA molecules which are complementary to a DNA template. Upon hybridization of a primer to the single-stranded DNA template, polymerases synthesize DNA in the 5' to 3' direction, successively adding nucleotides to the 3'-hydroxyl group of the growing strand. Thus, in the presence of deoxyribonucleoside triphosphates (dNTPs) and a primer, a new DNA molecule, complementary to the single stranded DNA template, can be synthesized.

A number of DNA polymerases have been isolated from mesophilic microorganisms such as *E. coli*. A number of these mesophilic DNA polymerases have also been cloned. Lin *et al.* cloned and expressed T4 DNA polymerase in *E. coli* (*Proc. Natl. Acad. Sci. USA* 84:7000-7004 (1987)). Tabor *et al.* (U.S. Patent No. 4,795,699) describes a cloned T7 DNA polymerase, while Minkley *et al.* (*J. Biol. Chem.* 259:10386-10392 (1984)) and Chatterjee (U.S. Patent No. 5,047,342) described *E. coli* DNA polymerase I and the cloning of T5 DNA polymerase, respectively.

Although DNA polymerases from thermophiles are known, relatively little investigation has been done to isolate and even clone these enzymes. Chien *et al.*, *J. Bacteriol.* 127:1550-1557 (1976) describe a purification scheme for obtaining a polymerase from *Thermus aquaticus* (Taq). The resulting protein had a molecular weight of about 63,000 daltons by gel filtration analysis and 68,000 daltons by sucrose gradient centrifugation. Kaledin *et al.*, *Biokhimiya* 45:644-51 (1980) disclosed a purification procedure for isolating DNA polymerase from *T. aquaticus* YT1 strain. The purified enzyme was reported to be a 62,000 dalton monomeric protein. Gelfand *et al.* (U.S. Patent No. 4,889,818) cloned a gene encoding a thermostable DNA polymerase from *Thermus aquaticus*. The molecular weight of this protein was found to be about 86,000 to 90,000 daltons.

Simpson *et al.* purified and partially characterized a thermostable DNA polymerase from a *Thermotoga* species (*Biochem. Cell. Biol.* 86:1292-1296 (1990)). The purified DNA polymerase isolated by Simpson *et al.* exhibited a

5'-exonuclease activity (Joyce *et al.*, *J. Biol. Chem.* 257:1958-64 (1990).) Polymerases lacking this activity are useful for DNA sequencing.

Most DNA polymerases also contain a 3'-5' exonuclease activity. This exonuclease activity provides a proofreading ability to the DNA polymerase. A T5 DNA polymerase that lacks 3'-5' exonuclease activity is disclosed in U.S. Patent No. 5,270,179. Polymerases lacking this activity are useful for DNA sequencing.

The polymerase active site, including the dNTP binding domain is usually present at the carboxyl terminal region of the polymerase (Ollis *et al.*, *Nature* 313:762-766 (1985); Freemont *et al.*, *Proteins* 1:66-73 (1986)). It has been shown that Phe⁷⁶² of *E. coli* polymerase I is one of the amino acids that directly interacts with the nucleotides (Joyce & Steitz, *Ann. Rev. Biochem.* 63:777-822 (1994); Astatke, *J. Biol. Chem.* 270:1945-54 (1995)). Converting this amino acid to a Tyr results in a mutant DNA polymerase that does not discriminate against dideoxynucleotides and is highly processive. See copending U.S. Application No. 08/525,087, of Deb K. Chatterjee, filed September 8, 1995, entitled "Mutant DNA Polymerases and the Use Thereof," which is expressly incorporated herein by reference.

Thus, there exists a need in the art to develop thermostable processive DNA polymerases. There also exists a need in the art to obtain wild type or mutant DNA polymerases that are devoid of exonuclease activities and are non-discriminating against dideoxynucleotides.

Summary of the Invention

The present invention satisfies these needs in the art by providing additional DNA polymerases useful in molecular biology. Specifically, this invention includes a thermostable DNA polymerase having a molecular weight of about 100 kilodaltons. More specifically, the DNA polymerase of the invention is isolated from *Thermotoga neapolitana* (Tne). The *Thermotoga* species preferred for isolating the DNA polymerase of the present invention was isolated

from an African continental solfataric spring (Windberger *et al.*, *Arch. Microbiol.* 151. 506-512, (1989)).

The Tne DNA polymerase of the present invention is extremely thermostable, showing more than 50% of activity after being heated for 60 minutes at 90°C with or without detergent. Thus, the DNA polymerase of the present invention is more thermostable than Taq DNA polymerase.

The present invention is also directed to cloning a gene encoding a *Thermotoga neapolitana* DNA polymerase enzyme. DNA molecules containing the Tne DNA polymerase gene, according to the present invention, can be transformed and expressed in a host cell to produce a Tne DNA polymerase having a molecular weight of 100 kilodaltons. Any number of hosts may be used to express the *Thermotoga* DNA polymerase gene of the present invention; including prokaryotic and eukaryotic cells. Preferably, prokaryotic cells are used to express the DNA polymerase of the invention. The preferred prokaryotic hosts according to the present invention is *E. coli*.

The Tne DNA polymerase of the invention may be used in well known DNA sequencing (dideoxy DNA sequencing, cycle DNA sequencing of plasmid DNAs, etc.) and DNA amplification reactions.

The present invention is also directed to mutant thermostable DNA polymerases. More specifically, the mutant DNA polymerases of the invention are derived from *Thermotoga neapolitana* and are substantially reduced or devoid of 3'-5' exonuclease activity, 5'-3' exonuclease activity, or is nondiscriminating against dideoxynucleotides. The present invention also relates to mutants having more than one of these properties, and DNA molecules containing the mutant Tne DNA polymerase enzyme genes. These mutants may also be used in well known DNA sequencing and DNA amplification reactions.

Detailed Description of the Preferred Embodiments

Definitions

In the description that follows, a number of terms used in recombinant DNA technology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Cloning vector. A plasmid, cosmid or phage DNA or other DNA molecule which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, are tetracycline resistance or ampicillin resistance.

Expression vector. A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences.

Recombinant host. Any prokaryotic or eukaryotic or microorganism which contains the desired cloned genes in an expression vector, cloning vector or any DNA molecule. The term "recombinant host" is also meant to include those host cells which have been genetically engineered to contain the desired gene on the host chromosome or genome.

Host. Any prokaryotic or eukaryotic microorganism that is the recipient of a replicable expression vector, cloning vector or any DNA molecule. The DNA molecule may contain, but is not limited to, a structural gene, a promoter and/or an origin of replication.

complementary to said template or a portion thereof. The newly synthesized DNA molecule, according to the invention, may be equal or shorter in length than the original DNA template. Mismatch incorporation during the synthesis or extension of the newly synthesized DNA molecule may result in one or a number of mismatched base pairs. Thus, the synthesized DNA molecule need not be exactly complementary to the DNA template.

Incorporating. The term "incorporating" as used herein means becoming a part of a DNA molecule or primer.

Amplification. As used herein "amplification" refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a DNA polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA molecule or primer thereby forming a new DNA molecule complementary to a DNA template. The formed DNA molecule and its template can be used as templates to synthesize additional DNA molecules. As used herein, one amplification reaction may consist of many rounds of DNA replication. DNA amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 30 to 100 "cycles" of denaturation and synthesis of a DNA molecule.

Oligonucleotide. "Oligonucleotide" refers to a synthetic or natural molecule comprising a covalently linked sequence of nucleotides which are joined by a phosphodiester bond between the 3' position of the pentose of one nucleotide and the 5' position of the pentose of the adjacent nucleotide.

Nucleotide. As used herein "nucleotide" refers to a base-sugar-phosphate combination. Nucleotides are monomeric units of a nucleic acid sequence (DNA and RNA). The term nucleotide includes deoxyribonucleoside triphosphates such as dATP, dCTP, dUTP, dGTP, dTTP, or derivatives thereof. Such derivatives include, for example, dm, [α S]dATP and 7-deaza-dGTP. The term nucleotide as used herein also refers to dideoxyribonucleoside triphosphates (ddNTPs) and their derivatives. Illustrated examples of dideoxyribonucleoside triphosphates include, but are not limited to, ddATP, ddCTP, ddGTP, ddITP, and ddTTP. According to the present invention, a "nucleotide" may be unlabeled or detectably labeled by

A "DNA polymerase substantially reduced in 3'-to-5' exonuclease activity" is defined herein as either (1) a mutated DNA polymerase that has about or less than 10%, or preferably about or less than 1%, of the 3'-to-5' exonuclease activity of the corresponding unmutated, wild-type enzyme, or (2) a DNA polymerase having a 3'-to-5' exonuclease specific activity which is less than about 1 unit/mg protein, or preferably about or less than 0.1 units/mg protein. A unit of activity of 3'-to-5' exonuclease is defined as the amount of activity that solubilizes 10 nmoles of substrate ends in 60 min. at 37°C, assayed as described in the "BRL 1989 Catalogue & Reference Guide", page 5, with *HhaI* fragments of *lambda* DNA 3'-end labeled with [³H]dTTP by terminal deoxynucleotidyl transferase (TdT). Protein is measured by the method of Bradford, *Anal. Biochem.* 72:248 (1976). As a means of comparison, natural, wild-type T5-DNAP or T5-DNAP encoded by pTTQ19-T5-2 has a specific activity of about 10 units/mg protein while the DNA polymerase encoded by pTTQ19-T5-2(Exo⁻) (U.S. 5,270,179) has a specific activity of about 0.0001 units/mg protein, or 0.001% of the specific activity of the unmodified enzyme, a 10⁵-fold reduction.

A "DNA polymerase substantially reduced in 5'-to-3' exonuclease activity" is defined herein as either (1) a mutated DNA polymerase that has about or less than 10%, or preferably about or less than 1%, of the 5'-to-3' exonuclease activity of the corresponding unmutated, wild-type enzyme, or (2) a DNA polymerase having 5'-to-3' exonuclease specific activity which is less than about 1 unit mg protein, or preferably about or less than 0.1 units/mg protein.

Both of these activities, 3'-to-5' exonuclease activity and 5'-to-3' exonuclease activity, can be observed on sequencing gels. Active 5'-to-3' exonuclease activity will produce nonspecific ladders in a sequencing gel by removing nucleotides from growing primers. 3'-to-5' exonuclease activity can be measured by following the degradation of radiolabeled primers in a sequencing gel. Thus, the relative amounts of these activities, e.g. by comparing wild-type and mutant polymerases, can be determined from these characteristics of the sequencing gel.

Igaki, (*Jpn. J. Bacteriol.* 33:729-742 (1978)). Broad-host range plasmids or cosmids, such as pCP13 (Darzins and Chakrabarty, *J. Bacteriol.* 159:9-18, 1984) can also be used for the present invention. The preferred vectors for cloning the genes of the present invention are prokaryotic vectors. Preferably, pCP13 and pUC vectors are used to clone the genes of the present invention.

The preferred host for cloning the wild type or mutant DNA polymerase genes of the invention is a prokaryotic host. The most preferred prokaryotic host is *E. coli*. However, the wild type or mutant DNA polymerase genes of the present invention may be cloned in other prokaryotic hosts including, but not limited to, *Escherichia*, *Bacillus*, *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, and *Proteus*. Bacterial hosts of particular interest include *E. coli* DH10B, which may be obtained from Life Technologies, Inc. (LTI) (Gaithersburg, MD).

Eukaryotic hosts for cloning and expression of the wild type or mutant DNA polymerases of the present invention include yeast, fungi, and mammalian cells. Expression of the desired DNA polymerase in such eukaryotic cells may require the use of eukaryotic regulatory regions which include eukaryotic promoters. Cloning and expressing the wild type or mutant DNA polymerase gene of the invention in eukaryotic cells may be accomplished by well known techniques using well known eukaryotic vector systems.

Once a DNA library has been constructed in a particular vector, an appropriate host is transformed by well known techniques. Transformed colonies are plated at a density of approximately 200-300 colonies per petri dish. Colonies are then screened for the expression of a heat stable DNA polymerase by transferring transformed *E. coli* colonies to nitrocellulose membranes. After the transferred cells are grown on nitrocellulose (approximately 12 hours), the cells are lysed by standard techniques, and the membranes are then treated at 95°C for 5 minutes to inactivate the endogenous *E. coli* enzyme. Other temperatures may be used to inactivate the host polymerases depending on the host used and the temperature stability of the DNA polymerase to be cloned. Stable DNA polymerase activity is then detected by assaying for the presence of DNA

nucleotides encoding the 219 amino terminal amino acids of Tne DNA polymerase. Examples of such a clone are pTTQTne535FY and pTTQTne5FY.

Tne DNA polymerase mutants can also be made to render the polymerase non-discriminating against non-natural nucleotides such as dideoxynucleotides. By way of example, one Tne DNA polymerase mutant having this property substitutes a Tyr for Phe at amino acid 67 as numbered in Figure 5. Other changes within the O helix of various polymerases such as other point mutations, deletions, and insertions can also be made.

B. Enhancing Expression of Thermotoga neapolitana DNA Polymerase

To optimize expression of the wild type or mutant *Thermotoga* DNA polymerases of the present invention, inducible or constitutive promoters are well known and may be used to express high levels of a polymerase structural gene in a recombinant host. Similarly, high copy number vectors, well known in the art, may be used to achieve high levels of expression. Vectors having an inducible high copy number may also be useful to enhance expression of *Thermotoga* DNA polymerase in a recombinant host.

To express the desired structural gene in a prokaryotic cell (such as, *E. coli*, *B. subtilis*, *Pseudomonas*, etc.), it is necessary to operably link the desired structural gene to a functional prokaryotic promoter. However, the natural *Thermotoga neapolitana* promoter may function in prokaryotic hosts allowing expression of the polymerase gene. Thus, the natural *Thermotoga* promoter or other promoters may be used to express the DNA polymerase gene. Such other promoters may be used to enhance expression and may either be constitutive or regulatable (i.e., inducible or derepressible) promoters. Examples of constitutive promoters include the *int* promoter of bacteriophage λ , and the *bla* promoter of the β -lactamase gene of pBR322. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (P_L and P_R), *trp*, *recA*, *lacZ*, *lacI*, *gal*, *trc*, and *tac* promoters of *E. coli*. The *B. subtilis* promoters include α -amylase (Ulmanen *et al.*, *J. Bacteriol* 162:176-182 (1985)) and *Bacillus*

(1986). Media formulations are also described in DSM or ATCC Catalogs and Sambrook *et al.*, In: *Molecular Cloning, A Laboratory Manual* (2nd ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

5 *Thermotoga neapolitana* and recombinant host cells producing the DNA polymerase of this invention can be separated from liquid culture, for example, by centrifugation. In general, the collected microbial cells are dispersed in a suitable buffer, and then broken down by ultrasonic treatment or by other well known procedures to allow extraction of the enzymes by the buffer solution. After removal of cell debris by ultracentrifugation or centrifugation, the DNA
10 polymerase can be purified by standard protein purification techniques such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis or the like. Assays to detect the presence of the DNA polymerase during purification are well known in the art and can be used during conventional biochemical purification methods to determine the presence of these enzymes.

15 **D. Uses of *Thermotoga neapolitana* DNA polymerase**

The wild type and mutant *Thermotoga neapolitana* DNA polymerases (Tne) of the present invention may be used in well known DNA sequencing, DNA labeling, and DNA amplification reactions. The DNA polymerase mutants devoid of or substantially reduced in 3'→5' exonuclease activity, devoid of or
20 substantially reduced in 5'→3' exonuclease activity, or containing a Phe⁶⁷→Tyr⁶⁷ mutation are especially useful for DNA sequencing, DNA labeling, and DNA amplification reactions. Moreover, Tne polymerase mutants containing two or more of these properties are also especially useful for DNA sequencing, DNA labeling, on DNA amplification reactions. As is well known, sequencing reactions
25 (dideoxy DNA sequencing and cycle DNA sequencing of plasmid DNA) require the use of DNA polymerases. Dideoxy-mediated sequencing involves the use of a chain-termination technique which uses a specific polymer for extension by DNA polymerase, a base-specific chain terminator and the use of polyacrylamide gels to separate the newly synthesized chain-terminated DNA molecules by size so that

polymerase of the present invention may be useful for incorporating α S nucleotides ([α S]dATP, [α S]dTTP, [α S]dCTP and [α S]dGTP) during sequencing (or labeling) reactions. For example, [α^{35} S]dATP, a commonly used detectably labeled nucleotide in sequencing reactions, is incorporated three times more efficiently with the Tne DNA polymerase of the present invention, than with Taq DNA polymerase. Thus, the enzyme of the present invention is particularly suited for sequencing or labeling DNA molecules with [α^{35} S]dNTPs.

Polymerase chain reaction (PCR), a well known DNA amplification technique, is a process by which DNA polymerase and deoxyribonucleoside triphosphates are used to amplify a target DNA template. In such PCR reactions, two primers, one complementary to the 3' termini (or near the 3'-termini) of the first strand of the DNA molecule to be amplified, and a second primer complementary to the 3' termini (or near the 3'-termini) of the second strand of the DNA molecule to be amplified, are hybridized to their respective DNA molecules. After hybridization, DNA polymerase, in the presence of deoxyribonucleoside triphosphates, allows the synthesis of a third DNA molecule complementary to the first strand and a fourth DNA molecule complementary to the second strand of the DNA molecule to be amplified. This synthesis results in two double stranded DNA molecules. Such double stranded DNA molecules may then be used as DNA templates for synthesis of additional DNA molecules by providing a DNA polymerase, primers, and deoxyribonucleoside triphosphates. As is well known, the additional synthesis is carried out by "cycling" the original reaction (with excess primers and deoxyribonucleoside triphosphates) allowing multiple denaturing and synthesis steps. Typically, denaturing of double stranded DNA molecules to form single stranded DNA templates is accomplished by high temperatures. The wild type and mutant *Thermotoga* DNA polymerases of the present invention are heat stable DNA polymerases, and thus will survive such thermal cycling during DNA amplification reactions. Thus, the wild type and mutant Tne DNA polymerases of the invention are ideally suited for PCR reactions, particularly where high temperatures are used to denature the DNA molecules during amplification.

Having now generally described the invention, the same will be more readily understood through reference to the following Examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

5 ***Example 1: Bacterial Strains And Growth Conditions***

Thermotoga neapolitana DSM No. 5068 was grown under anaerobic conditions as described in the DSM catalog (addition of resazurin, Na₂S, and sulfur granules while sparging the media with nitrogen) at 85°C in an oil bath from 12 to 24 hours. The cells were harvested by filtering the broth through Whatman
10 #1 filter paper. The supernatant was collected in an ice bath and then centrifuged in a refrigerated centrifuge at 8,000 rpms for twenty minutes. The cell paste was stored at -70°C prior to total genomic DNA isolation.

E. coli strains were grown in 2X LB broth base (Lennox L broth base: GIBCO/BRL) medium. Transformed cells were incubated in SOC (2% tryptone,
15 0.5% yeast extract, yeast 10 mM NaCl, 2.5 M KCl, 20mM glucose, 10mM MgCl₂, and 10mM MgSO₄ per liter) before plating. When appropriate antibiotic supplements were 20 mg/l tetracycline and 100 mg/l ampicillin. *E. coli* strain DH10B (Lorow *et al.*, *Focus* 12:19-20 (1990)) was used as host strain. Competent DH10B may be obtained from Life Technologies, Inc. (LTI)
20 (Gaithersburg, MD).

Example 2: DNA Isolation

Thermotoga neapolitana chromosomal DNA was isolated from 1.1g of cells by suspending the cells in 2.5 ml TNE (50mM Tris-HCl, pH 8.0, 50mM NaCl, 10mM EDTA) and treated with 1% SDS for 10 minutes at 37°C. DNA
25 was extracted with phenol by gently rocking the lysed cells overnight at 4°C. The next day, the lysed cells were extracted with chloroform:isoamyl alcohol. The resulting chromosomal DNA was further purified by centrifugation in a CsCl

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fumes of chloroform:toluene (1:1) for 20 minutes and dried for 10 minutes at room temperature. The membranes were then treated at 95°C for 5 minutes to inactivate the endogenous *E. coli* enzymes. Surviving DNA polymerase activity was detected by submerging the membranes in 15 ml of polymerase reaction mix

5 (50 mM Tris-HCl (pH 8.8), 1 mM MgCl₂, 3 mM β-mercaptoethanol, 10 μM dCTP, dGTP, dTTP, and 15 μCi of 3,000 Ci/mmol [α^{32} P]dATP) for 30 minutes at 65°C.

Using autoradiography, three colonies were identified that expressed a *Thermotoga neapolitana* DNA polymerase. The cells were grown in liquid

10 culture and the protein extract was made by sonication. The presence of the cloned thermostable polymerase was confirmed by treatment at 90°C followed by measurement of DNA polymerase activity by incorporation of radioactive deoxyribonucleoside triphosphates into acid insoluble DNA. One of the clones, expressing Tne DNA polymerase, contained a plasmid designated pCP13-32 was

15 used for further study.

Example 5: Subcloning of Tne DNA polymerase

Since the pCP13-32 clone expressing the Tne polymerase gene contains about 25 kb of *T. neapolitana* DNA, we attempted to subclone a smaller fragment of the Tne polymerase gene. The molecular weight of the Tne polymerase purified

20 from *E. coli*/pCP13-32 was about 100 kD. Therefore, a 2.5-3.0 kb DNA fragment will be sufficient to code for full-length polymerase. A second round of *Sau*3A partial digestion similar to Example 3 was done using pCP13-32 DNA. In this case, a 3.5 kb region was cut out from the agarose gel, purified by Gene Clean (BIO 101, La Jolla, CA) and ligated into plasmid pSport 1 (Life Technologies,

25 Inc.) which had been linearized with *Bam*HI and dephosphorylated with calf intestinal phosphatase. After ligation, DH10B was transformed and colonies were tested for DNA polymerase activity as described in Example 4. Several clones were identified that expressed Tne DNA polymerase. One of the clones (pSport-Tne) containing about 3 kb insert was further characterized. A restriction

fractions were dialyzed against 20 volumes of column buffer. The pooled fractions were applied to a Toso650Q column (Tosohaas). The column was washed to baseline OD₂₈₀ and elution effected with a linear 10 column volume gradient of 25 mM Tris, pH 7.4, 8% glycerol, 0.5 mM EDTA, 10 mM KCl, 5 mM β -mercaptoethanol to the same buffer plus 650 mM KCl. Active fractions were pooled.

Example 7: Characterization of Purified The DNA Polymerase

1. Determination of the Molecular Weight of *Thermotoga neapolitana* DNA Polymerase

The molecular weight of 100 kilodaltons was determined by electrophoresis in a 12.5% SDS gel by the method of Laemmli, U.K., *Nature* (Lond.) 227:680-685 (1970). Proteins were detected by staining with Coomassie brilliant blue. A 10 kd protein ladder (Life Technologies, Inc.) was used as standard.

2. Method for Measuring Incorporation of [α^{35} S]-dATP Relative to 3 H-dATP

Incorporation of [α S]dATP was evaluated in a final volume of 500 μ l of reaction mix, which was preincubated at 72°C for five minutes, containing either a [3 H]TTP nucleotide cocktail (100 μ M each TTP, dATP, dCTP, dGTP with [3 H]TTP at 90.3 cpm/pmol), a nucleotide cocktail containing [α S]dATP as the only source of dATP (100 μ M each [α S]dATP, dCTP, dGTP, TTP with [α^{35} S]dATP at 235 cpm/pmol), or a mixed cocktail (50 μ M [α S]dATP, 50 μ M dATP, 100 μ M TTP, 100 μ M dCTP, 100 μ M dGTP with [$^{35}\alpha$ S] dATP at 118 cpm/pmol and [3 H]TTP at 45.2 cpm/pmol). The reaction was initiated by the addition of 0.3 units of *T. neapolitana* DNA polymerase or *T. aquaticus* DNA polymerase. At the times indicated a 25 μ l aliquot was removed and quenched by addition of ice cold EDTA to a final concentration of 83 mM. 20 μ l aliquots of

mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, and 100 μM [³H]dGTP (132 cpm/pmole), or 100 mM KCl, 0.5 mM MnCl₂, and 200 μM [³H]dGTP (107 cpm/pmole). Reaction mixtures also contained either 2.5 units of the Tth DNA polymerase (Perkin-Elmer) or 2.5 units of the Tne DNA polymerase. Incubations were at 45°C for 10 min followed by 75°C for 20 min.

The table shows the results of determining the relative levels of incorporation of Tne and Tth DNA polymerase with (A)_n:(dT)₂₀ and (C)_n:(dG)₁₂₋₁₈ in the presence of Mg⁺⁺ and Mn⁺⁺. Tne DNA polymerase appears to be a better reverse transcriptase than Tth DNA polymerase under reaction conditions more specific for reverse transcriptase, i.e., in the presence of (A)_n:(dT)₂₀ with Mg⁺⁺ and (C)_n:(dG)₁₂₋₁₈ with Mn⁺⁺ or Mg⁺⁺.

**DNA Polymerase Activity of Tth and Tne
DNA Polymerase with (A)_n:(dT)₂₀ and (C)_n:(dG)₁₂₋₁₈**

Enzyme	DNA Polymerase Activity (pMoles Complementary [³ H]dNTP Incorporated)			
	(A) _n :(dT) ₂₀ Mg ⁺⁺ Mn ⁺⁺		(C) _n :(dG) ₁₂₋₁₈ Mg ⁺⁺ Mn ⁺⁺	
Tne	161.8	188.7	0.6	4.2
Tth	44.8	541.8	0	0.9

Example 9: Construction of Thermotoga Neapolitana 3'-to-5' Exonuclease Mutant

The amino acid sequence of portions of the Tne DNA polymerase was compared with other known DNA polymerases such as *E. coli* DNA polymerase 1, Taq DNA polymerase, T5 DNA polymerase, and T7 DNA polymerase to localize the regions of 3'-to-5' exonuclease activity, and the dNTP binding domains within the DNA polymerase. We have determined that one of the 3'-to-5' exonuclease domains based on the comparison of the amino acid sequences of

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(3'→5'), was isolated. The presence of the mutant sequence was confirmed by the presence of the unique *Eco47III* site. The plasmid was then digested with *SstI* and *HindIII*. The entire mutant polymerase gene (2.6 kb) was purified and cloned into *SstI* and *HindIII* digested pTrc99 expression vector (Pharmacia, Sweden). The clones were selected in DH10B (LTI, Gaithersburg, MD). The resulting plasmid was designated pTrcTne35. See Figure 6B. This clone produced active heat stable DNA polymerase.

Example 10: Phenylalanine to Tyrosine Mutant

As discussed *supra*, the polymerase active site including the dNTP binding domain is usually present at the carboxyl terminal region of the polymerase. The preliminary and partial sequence of the Tne polymerase gene suggests that the amino acids that presumably contact and interact with the dNTPs are present within the 694 bases starting at the internal *BamHI* site. See Figure 4. This conclusion is based on homology with a prototype polymerase *E. coli* polymerase 1. See Polisky et al., *J. Biol. Chem.* 265:14579-14591 (1990). The sequence of the carboxyl terminal portion of the polymerase gene is shown in Figure 5. Based upon this sequence, it is possible to compare the amino acid sequence within the O-helix for various polymerases:

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*

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Tne	63	KMVNFSIIYG	72	(SEQ ID NO. 9)
Pol I	758	KAINFGLIYG	767	(SEQ ID NO. 10)
T5	566	KAITFGILYG	575	(SEQ ID NO. 11)
T7	522	KTFIYGFLYG	531	(SEQ ID NO. 12)
Taq	663	KTINFGVLYG	672	(SEQ ID NO. 13)

30

It was shown that by replacing the phenylalanine residue of Taq DNA polymerases, (indicated as * above) the polymerase becomes non-discriminating against non-natural nucleotides such as dideoxynucleotides. See application Serial No. 08/525,087 entitled "Mutant DNA Polymerases and Use Thereof" of Deb K. Chatterjee, filed September 8, 1995, specifically incorporated herein by reference.

the smallest *Bam*HI fragment (0.8 kb) containing the Phe⁶⁷→Tyr⁶⁷ mutation was purified and ligated to generate pUC-Tne35FY. The proper orientation and the presence of both mutations in the same plasmid was confirmed by *Eco*47III, *Hpa*I, and *Sph*I-*Hind*III restriction digests. See Figure 7.

5 The entire polymerase containing both mutations was subcloned as a *Sst*I-*Hind*III fragment in pTrc99 to generate pTrcTne35FY in DH10B. The clone produced active heat stable polymerase.

***Example 12: 3'-to-5' Exonuclease, 5'-to-3' Exonuclease, and
Phe⁶⁷→Tyr⁶⁷ Triple Mutants***

10 In most of the known polymerases, the 5'-to-3' exonuclease activity is present at the amino terminal region of the polymerase (Ollis, D.L., *et al.*, *Nature* 313, 762-766, 1985; Freemont, P.S., *et al.*, *Proteins* 1, 66-73, 1986; Joyce, C.M., *Curr. Opin. Struct. Biol.* 1, 123-129, 1991). There are some conserved amino acids that are implicated to be responsible for 5'-to-3' exonuclease activity
15 (Gutman and Minton, *Nucl. Acids Res.* 21, 4406-4407, 1993). *See supra*. It is known that 5'-to-3' exonuclease domain is dispensable. The best known example is the Klenow fragment of *E. coli* Pol I. The Klenow fragment is a natural proteolytic fragment devoid of 5'-to-3' exonuclease activity (Joyce, C.M., *et al.*, *J. Biol. Chem.* 257, 1958-1964, 1990). In order to generate an equivalent mutant
20 for Tne DNA polymerase devoid of 5'-to-3' exonuclease activity we exploited the presence of a unique *Sph*I site present 680 bases from the *Sst*I site. pUC-Tne35FY was digested with *Hind*III, filled-in with Klenow fragment to generate a blunt-end, and digested with *Sph*I. The 1.9 kb fragment was cloned into an expression vector pTTQ19 (Stark, M.J.R., *Gene* 51, 255-267, 1987) at the
25 *Sph*I-*Sma*I sites and was introduced into DH10B. This cloning strategy generated an in-frame polymerase clone with an initiation codon for methionine from the vector. The resulting clone is devoid of 219 amino terminal amino acids of Tne DNA polymerase. This clone is designated as pTTQTne535FY. The clone produced active heat stable polymerase. No exonuclease activity could be

Example 14: Purification of the Mutant Polymerases

The purification of the mutant polymerases was done essentially as described in U.S. Patent Application Serial No. 08/370,190, filed January 9, 1995, entitled "Cloned DNA Polymerases for *Thermotoga neapolitana*," and as in Example 6, *supra*, with minor modifications. Specifically, 5 to 10 grams of cells expressing cloned mutant Tne DNA polymerase were lysed by sonication with a Heat Systems Ultrasonic, Inc. Model 375 machine in a sonication buffer comprising 50 mM Tris-HCl, pH 7.4; 8% glycerol; 5 mM 2-mercaptoethanol, 10 mM NaCl, 1 mM EDTA, and 0.5 mM PMSF. The sonication sample was heated at 75°C for 15 minutes. Following heat treatment, 200 mM NaCl and 0.4% PEI was added to remove nucleic acids. The extract was centrifuged for clarification. Ammonium sulfate was added to 48%, the pellet was resuspended in a column buffer consisting of 25 mM Tris-HCl, pH 7.4; 8% glycerol; 0.5% EDTA; 5 mM 2-mercaptoethanol; 10 mM KCl and loaded on a Heparin agarose column. The column was washed with 10 column volumes using the loading buffer and eluted with a 10 column volume buffer gradient from 10 mM to 1 M KCl. Fractions containing polymerase activity were pooled and dialyzed in column buffer as above with the pH adjusted to 7.8. The dialyzed pool of fractions were loaded onto a mono Q column. The column was washed and eluted as described above for the Heparin column. The active fractions are pooled and a unit assay was performed.

The unit assay reaction mixture contained 25 mM TAPS pH 9.3, 2 mM MgCl₂, 50 mM KCl, 1 mM DTT, 0.2 mM dNTPs, 500 µg/ml DNase I treated salmon sperm DNA, 21 mCi/ml [α P³²] dCTP and various amounts of polymerase in a final volume of 50 µl. After 10 minutes incubation at 70°C, 10 µl of 0.5 M EDTA was added to the tube. TCA precipitable counts were measured in GF/C filters using 40 µl of the reaction mixture.

- 35 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: LIFE TECHNOLOGIES, INC.
- (ii) TITLE OF INVENTION: Cloned DNA Polymerases from Thermotoga Neapolitana and Mutants Thereof
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: STERNE, KESSLER, GOLDSTEIN & FOX
 - (B) STREET: 1100 New York Avenue, N.W.
 - (C) CITY: Washington
 - (D) STATE: DC
 - (E) COUNTRY: USA
 - (F) ZIP: 20005
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: TO BE ASSIGNED
 - (B) FILING DATE: 02-OCT-1995
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/316,423
 - (B) FILING DATE: 30-SEP-1994
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/370,190
 - (B) FILING DATE: 09-JAN-1995
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Esmond, Robert W.
 - (B) REGISTRATION NUMBER: 32,893
 - (C) REFERENCE/DOCKET NUMBER: 0942.280PC02
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 202-371-2600
 - (B) TELEFAX: 202-371-2540

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:

- 37 -

ATGAAATACA GAGGATACGA CAAGAGAAAA CTACTTCCGA TATTGAAAGA ACTGGAGTTT	840
GCTTCCATCA TGAAGGAACT TCAACTGTAC GAAGAAGCAG AACCCACCGG ATACGAAATC	900
GTGAAGGATC ATAAGACCTT CGAAGATCTC ATCGAAAAGC TGAAGGAGGT TCCATCTTTT	960
GCCCTGGACC TTGAAACGTC CTCCTTGGAC CCGTTCAACT GTGAGATAGT CGGCATCTCC	1020
GTGTGTTTCA AACCGAAAAC AGCTTATTAC ATTCCAATTC ATCACAGAAA CGCCCACAAT	1080
CTTGATGAAA CACTGGTGCT GTCGAAGTTG AAAGAGATCC TCGAAGACCC GTCTTCGAAG	1140
ATTGTGGGTC AGAACCTGAA GTACGACTAC AAGGTTCTTA TGGTAAAGGG TATATCGCCA	1200
GTTTATCCGC ATTTTGACAC GATGATAGCT GCATATTTGC TGGAGCCAAA CGAGAAAAAA	1260
TTCAATCTCG AAGATCTGTC TTTGAAATTT CTCGGATACA AAATGACGTC	1310

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 436 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Ala	Arg	Leu	Phe	Leu	Phe	Asp	Gly	Thr	Ala	Leu	Ala	Tyr	Arg	Ala	1	5	10	15
Tyr	Tyr	Ala	Leu	Asp	Arg	Ser	Leu	Ser	Thr	Ser	Thr	Gly	Ile	Pro	Thr	20	25	30	
Asn	Ala	Val	Tyr	Gly	Val	Ala	Arg	Met	Leu	Val	Lys	Phe	Ile	Lys	Glu	35	40	45	
His	Ile	Ile	Pro	Glu	Lys	Asp	Tyr	Ala	Ala	Val	Ala	Phe	Asp	Lys	Lys	50	55	60	
Ala	Ala	Thr	Phe	Arg	His	Lys	Leu	Leu	Val	Ser	Asp	Lys	Ala	Gln	Arg	65	70	75	80
Pro	Lys	Thr	Pro	Ala	Leu	Leu	Val	Gln	Gln	Leu	Pro	Tyr	Ile	Lys	Arg	85	90	95	
Leu	Ile	Glu	Ala	Leu	Gly	Phe	Lys	Val	Leu	Glu	Leu	Glu	Gly	Tyr	Glu	100	105	110	

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Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Glu Pro
405 410 415

Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser Leu Lys Phe Leu Gly
420 425 430

Tyr Lys Met Thr
435

10. The recombinant host of claim 9, wherein said gene is obtained from *Thermotoga neapolitana* DSM 5068.

11. The recombinant host of claim 8, wherein said host is prokaryotic.

12. The recombinant host of claim 11, wherein said host is *E. coli*.

5 13. A method of producing a Tne DNA polymerase having a molecular weight of about 100 kilodaltons, said method comprising:

(a) culturing a cellular host comprising a gene encoding said DNA polymerase;

(b) expressing said gene; and

10 (c) isolating said DNA polymerase from said host.

14. The method of claim 13, wherein said host is a eukaryotic host.

15. The method of claim 13, wherein said host is a prokaryotic host.

16. The method of claim 15, wherein said prokaryotic host is *E. coli*.

15 17. A method of synthesizing a double-stranded DNA molecule comprising:

(a) hybridizing a primer to a first DNA molecule; and

(b) incubating said DNA molecule of step (a) in the presence of one or more deoxyribonucleoside triphosphates and Tne DNA polymerase having a molecular weight of about 100 kilodaltons, under conditions sufficient to synthesize a second DNA molecule complementary to all or a portion of said first DNA molecule.

20

18. The method of claim 17, wherein said DNA polymerase is isolated from *Thermotoga neapolitana*.

(c) incubating the mixture of step (b) under conditions sufficient to synthesize a random population of DNA molecules complementary to said first DNA molecule, wherein said synthesized DNA molecules are shorter in length than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 5' termini; and (d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

10 28. The method of claim 27, wherein said terminator nucleotide is ddTTP.

 29. The method of claim 27, wherein said terminator nucleotide is ddATP.

 30. The method of claim 27, wherein said terminator nucleotide is ddGTP.

15 31. The method of claim 27, wherein said terminator nucleotide is ddCTP.

 32. The method of claim 27, wherein said DNA polymerase is isolated from *Thermotoga neapolitana*.

20 33. The method of claim 32, wherein said DNA polymerase is isolated from *Thermotoga neapolitana* DSM 5068.

 34. The method of claim 27, wherein said DNA polymerase is isolated from a recombinant host expressing a gene encoding said DNA polymerase.

(a) a first container means comprising a Tne DNA polymerase having a molecular weight of about 100 kilodaltons;

(b) a second container means comprising one or more dideoxynucleoside triphosphates; and

5 (c) a third container means comprising one or more deoxyribonucleoside triphosphates.

42. The kit of claim 41, wherein said DNA polymerase is isolated from *Thermotoga neapolitana*.

10 43. The kit of claim 42, wherein said DNA polymerase is isolated from *Thermotoga neapolitana* DSM 5068.

44. The kit of claim 41, wherein said DNA polymerase is isolated from a recombinant host expressing a gene encoding said DNA polymerase.

45. A kit for amplifying a DNA molecule, comprising:

15 (a) a first container means comprising a Tne DNA polymerase having a molecular weight of about 100 kilodaltons; and

(b) a second container means comprising one or more deoxyribonucleoside triphosphates.

46. The kit of claim 45, wherein said DNA polymerase is isolated from *Thermotoga neapolitana*.

20 47. The kit of claim 46, wherein said DNA polymerase is isolated from *Thermotoga neapolitana* DSM 5068.

48. The kit of claim 45, wherein said DNA polymerase is isolated from a recombinant host expressing a gene encoding said DNA polymerase.

DNA polymerase; and (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against dideoxynucleotides, or fragments thereof.

5 56. The isolated DNA molecule as claimed in claim 55, wherein said DNA molecule is selected from the group consisting of pTrcTne35, pTrcTneFY, pTrcTne35FY, and pTTQTne535FY.

 57. The isolated DNA molecule as claimed in claim 55, wherein said DNA molecule further comprises expression control elements.

10 58. The isolated DNA molecule as claimed in claim 57, wherein said expression control elements comprise an inducible promoter selected from the group consisting of λP_L promoter, a tac promoter, a trp promoter, and a trc promoter.

15 59. A recombinant host comprising a DNA sequence encoding a mutant *Thermotoga neapolitana* DNA polymerase having at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3'-5' exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or eliminates 5'-3' exonuclease activity of said DNA polymerase; and (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against
20 dideoxynucleotides, or fragments thereof.

 60. A method of producing a Tne DNA polymerase, said method comprising:

25 (a) culturing a cellular host comprising a gene encoding a mutant *Thermotoga neapolitana* DNA polymerase having at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3'-5' exonuclease activity of said DNA polymerase; (2) a second

dUTP, ddATP, ddCTP, ddGTP, ddITP, ddTTP, [α S]dATP, [α S]dTTP, [α S]dGTP, and [α S]dCTP.

5 64. The method of synthesizing a double-stranded DNA molecule as claimed in claim 63, wherein one or more of said deoxyribonucleoside triphosphates are detectably labelled.

65. The method of synthesizing a double-stranded DNA molecule as claimed in claim 64, wherein said label is selected from the group consisting of a radioactive isotope, a fluorescent label, a chemiluminescent label, a bioluminescent label, and an enzyme label.

10 66. A method of sequencing a DNA molecule, comprising:
 (a) hybridizing a primer to a first DNA molecule;
 (b) contacting said DNA molecule of step (a) with deoxyribonucleoside triphosphates, a mutant *Thermotoga neapolitana* DNA polymerase, and a terminator nucleotide;
15 (c) incubating the mixture of step (b) under conditions sufficient to synthesize a random population of DNA molecules complementary to said first DNA molecule;

20 wherein said synthesized DNA molecules are shorter in length than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 5' termini; and

 (d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

25 wherein said mutant *Thermotoga neapolitana* DNA polymerase has at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3'-5' exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or eliminates 5'-3' exonuclease activity of said DNA polymerase; and (3) a third mutation in the O

(a) a first container means comprising a mutant *Thermotoga neapolitana* DNA polymerase;

(b) a second container means comprising one or more dideoxynucleoside triphosphates; and

(c) a third container means comprising one or more deoxyribonucleoside triphosphates,

wherein:

said mutant *Thermotoga neapolitana* DNA polymerase has at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3'→5' exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or eliminates 5'→3' exonuclease activity of said DNA polymerase; and (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against dideoxynucleotides.

70. A kit for amplifying a DNA molecule, comprising:

(a) a first container means comprising a mutant *Thermotoga neapolitana* DNA polymerase; and

(b) a second container means comprising one or more deoxyribonucleoside triphosphates,

wherein:

said mutant *Thermotoga neapolitana* DNA polymerase has at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3'→5' exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or eliminates 5'→3' exonuclease activity of said DNA polymerase; and (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against dideoxynucleotides.

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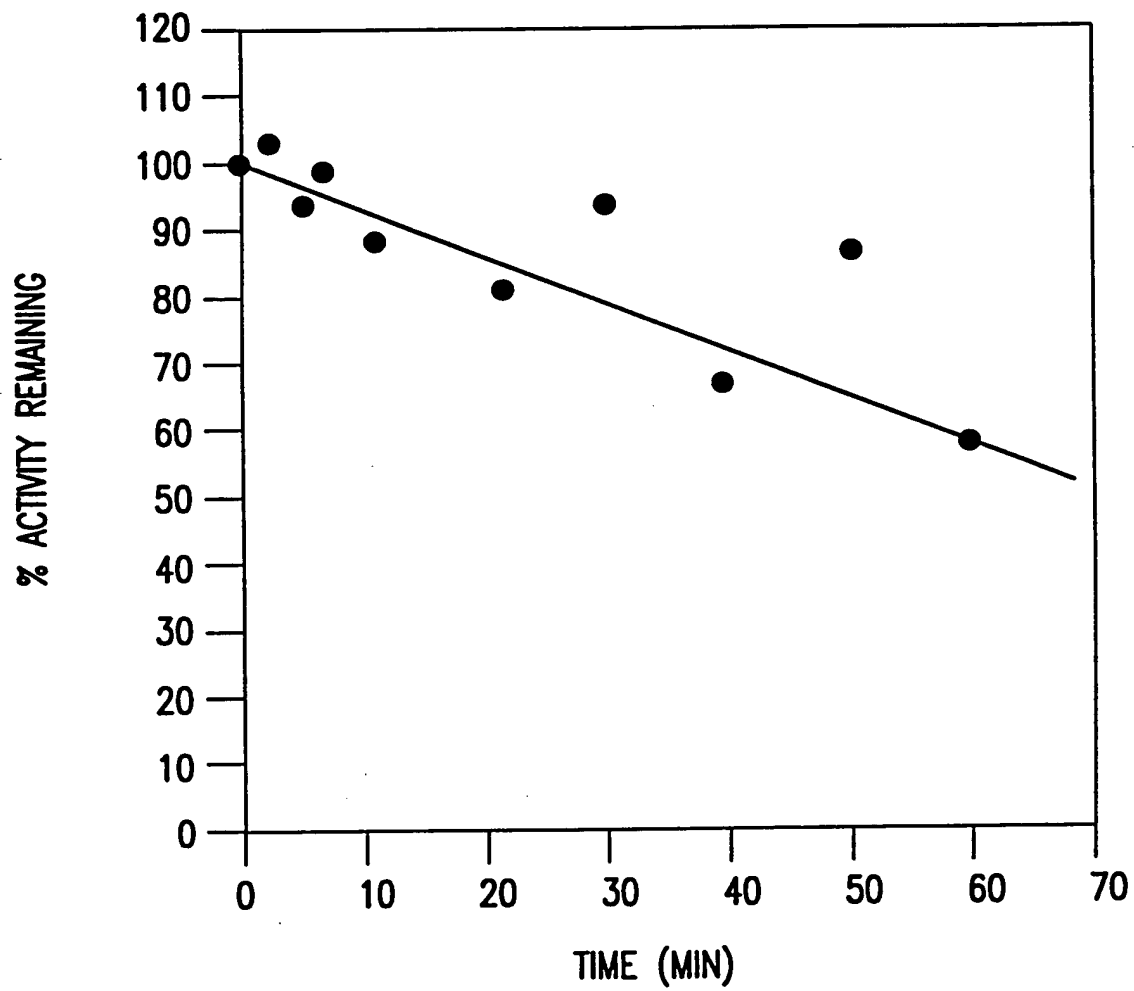


FIG.1

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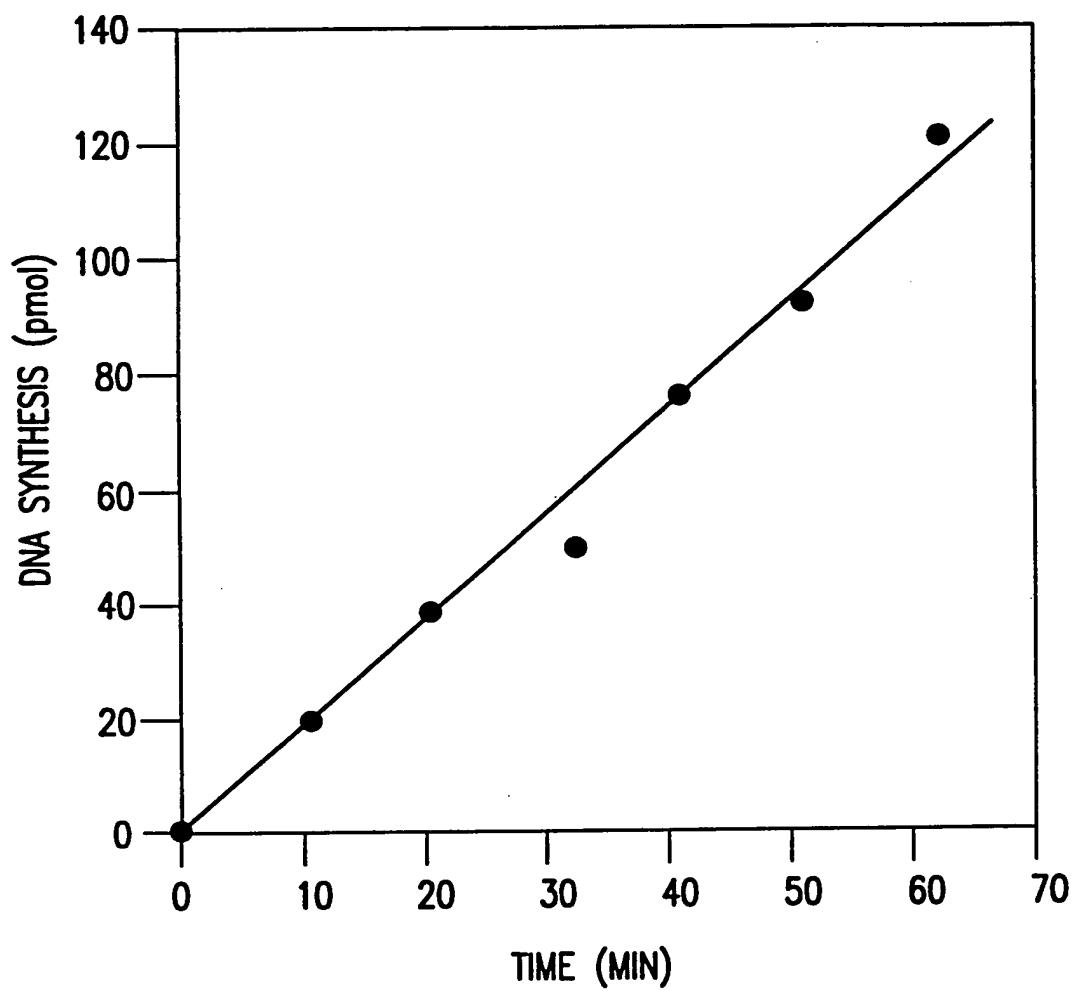


FIG.2

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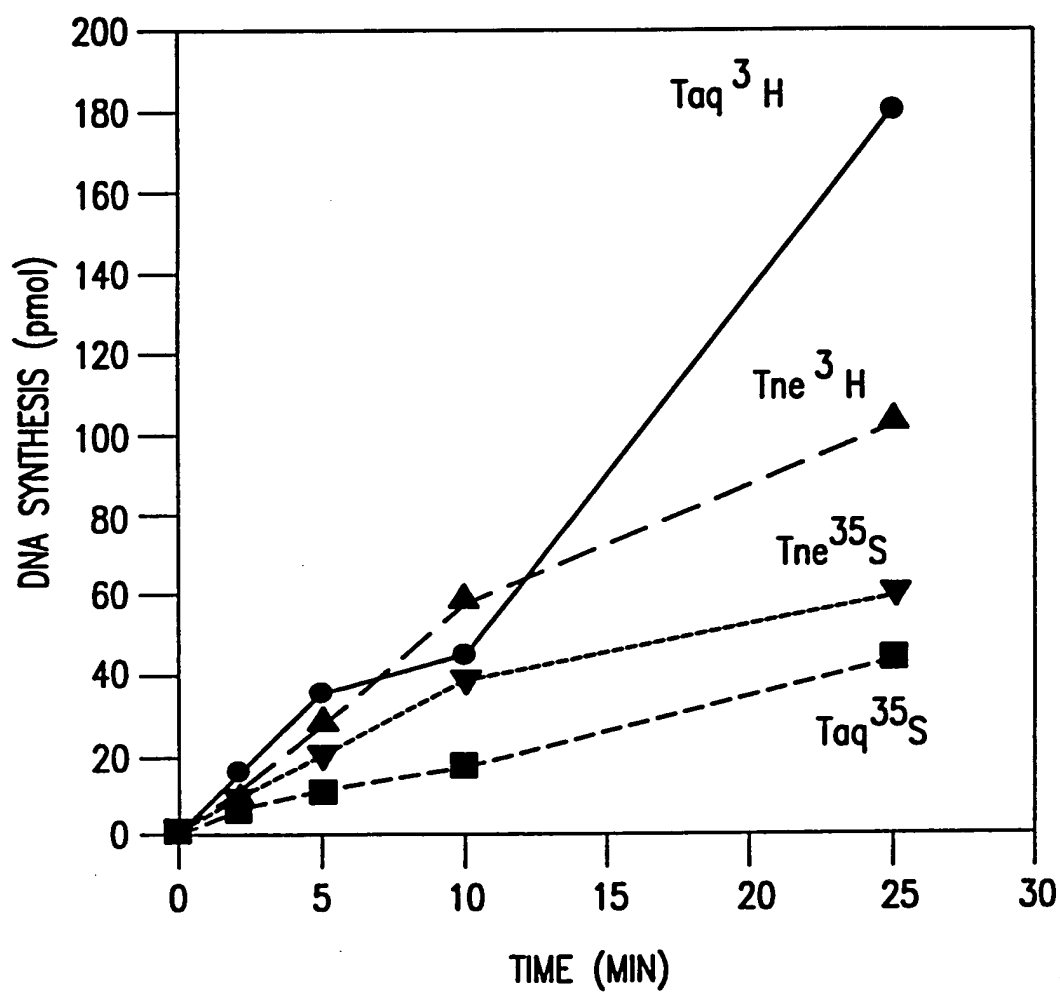


FIG.3

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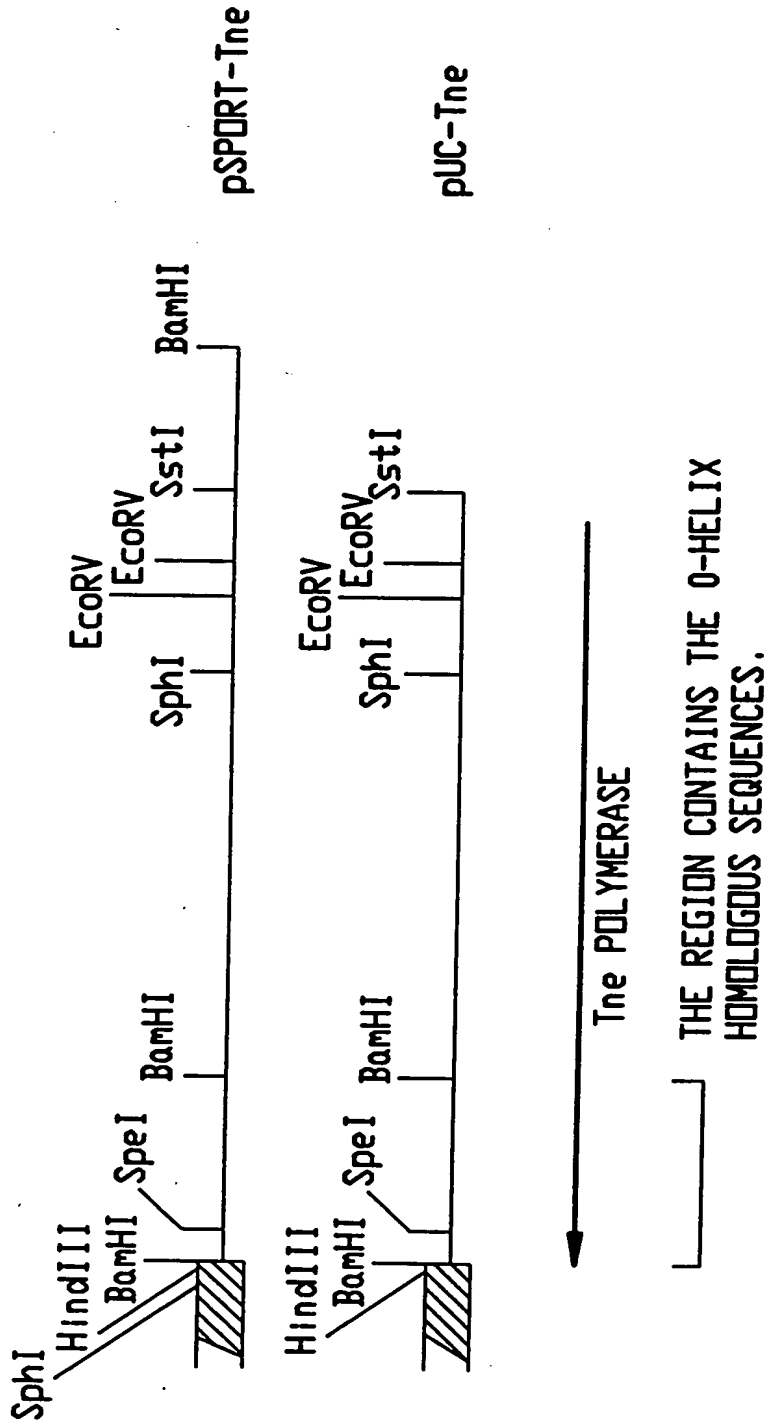


FIG.4

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BamHI

1 GGATCCAGAC TGGTGGATCG TCAGTGGCGA TTATTCCCAA ATACAACTCA GAATCCTCGC
G S R L V D R Q C G L F P N R T Q N P C
—▶ D P D W W I V S A D Y S Q I E L R I L
I Q T G G S S V R I I P K - N S E S S

61 TCATCTCAGT GGTGATGAGA ACCTTGTGAA GGCCTTCGAG GAGGGCATCG ATGTGCACAC
S S Q W - - E P C E G L R G G H R C A H
—▶ A H L S G D E N L V K A F E E G I D V H
L I S V V M R T L - R P S R R A S M C T

121 CTTGACTGCC TCCAGGATCT ACAACGTAAA GCCAGAAGAA GTGAACGAAG AAATCGGACG
L D C L Q D L Q R K A R R S E R R N A T
—▶ T L T A S R I Y N V K P E E V N E E M R
P - L P P G S T T - S Q K K - T K K C D

181 GGTGGAAAG ATGGTGAAC TCTCTATAAT ATACGGTGTC ACACCGTACG GTCTTTCTGT
G W K D G E L L Y N I R C H T V R S F C
—▶ R V G K M V N (F) S I I Y G V T P Y G L S
G L E R W - T S L - Y T V S H R T V F L

241 GAGACTTGA ATACCGGTTA AAGAAGCAGA AAAGATGATT ATCAGCTATT TCACACTGTA
E T W N T G - R S R K D D Y Q L F H T V
—▶ V R L G I P V K E A E K M I I S Y F T L
- D L E Y R L K K Q K R - L S A I S H C

301 TCCAAAGGTG CGAAGCTACA TCCAGCAGGT TGTTCAGAG GCAAAAGAGA AGGGCTACGT
S K G A K L H P A G C C R G K R E G L R
—▶ Y P K V R S Y I Q Q V V A E A K E K G Y
I Q R C E A T S S R L L Q R Q K R R A T

361 CAGGACTCTC TTTGGAAGAA AAAGAGATAT TCCCCAGCTC ATGGCAAGGG ACAAGAACAC
Q D S L W K K K R Y S P A H G K G Q E H
—▶ V R T L F G R K R D I P Q L M A R D K N
S G L S L E E K E I F P S S W Q G T R T

421 CCAGTCCGAA GGCGAAAGAA TCGCAATAAA CACCCCCATT CAGGGAAC TG CGGCAGATAT
P V R R R K N R N K H P H S G N C G R Y
—▶ T Q S E G E R I A I N T P I Q G T A A D
P S P K A K E S Q - T P P F R E L R Q I

FIG.5A

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481 AATAAAATTG GCTATGATAG ATATAGACGA GGAGCTGAGA AAAAGAAACA TGAAATCCAG
N K I G Y D R Y R R G A E K K K H E I Q
→ I I K L A M I D I D E E L R K R N M K S
- - N W L - - I - T R S - E K E T - N P

541 AATGATCATT CAGGTTCATG ACGAACTGGT CTTCGAGGTT CCCGATGAGG AAAAGAAGA
N D H S G S - R T G L R G S R - G K R R
→ R M I I Q V H D E L V F E V P D E E K E
E - S F R F M T N W S S R F P M R K K K

601 ACTAGTTGAT CTGGTGAAGA ACAAATGAC AAATGTGGTG AACTCTCTG TGCCTCTTGA
T S - S G E E Q N D K C G E T L C A S -
→ E L V D L V K N K M T N V V K L S V P L
N - L I W - R T K - Q M W - N S L C L L

661 GGTGACATA AGGATGGGAA AAAGCTGGTC TTGA
G - H K H R K K L V L
→ E V D I S I G K S W S -
R L T - A S E K A G L

FIG.5B

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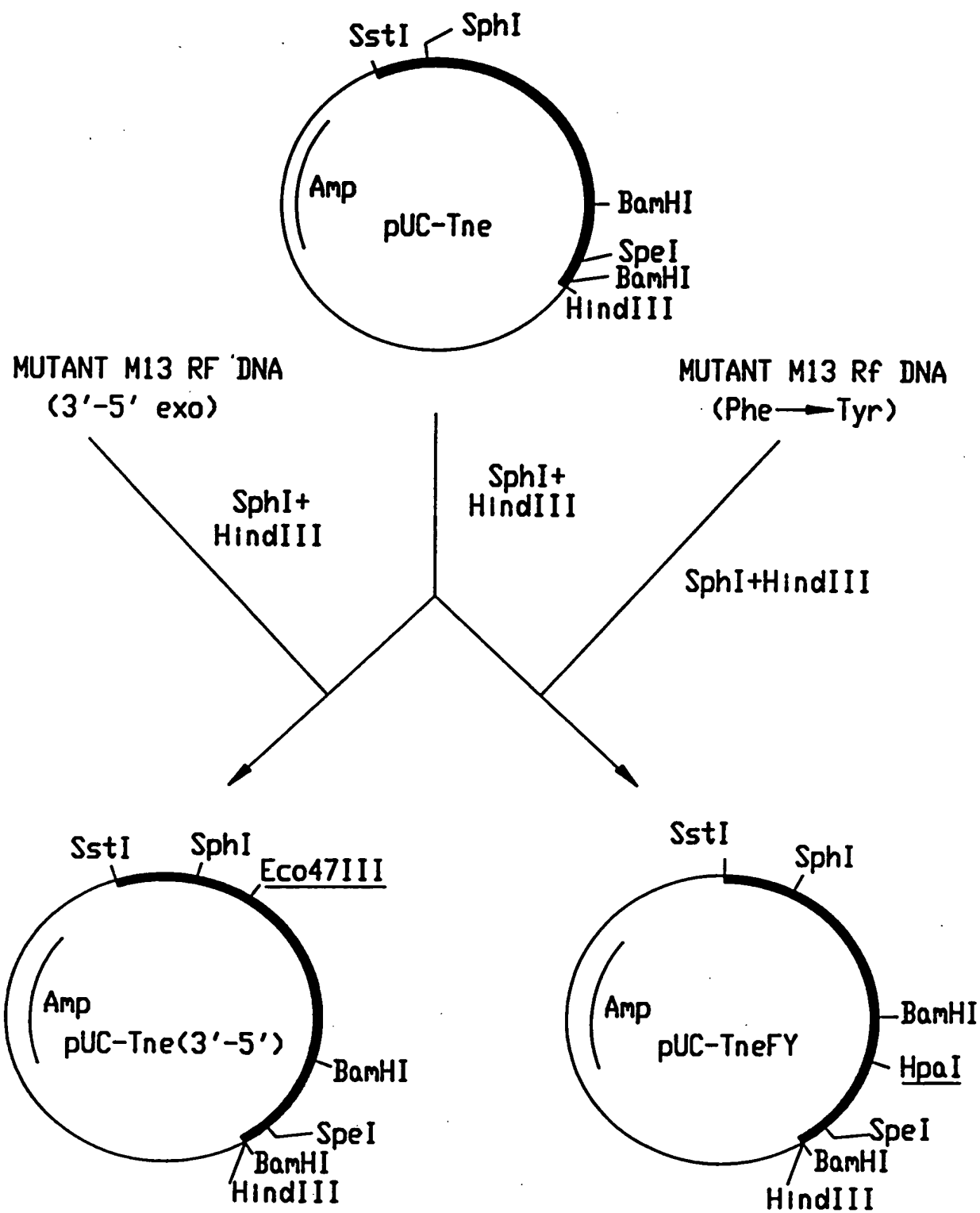


FIG. 6A

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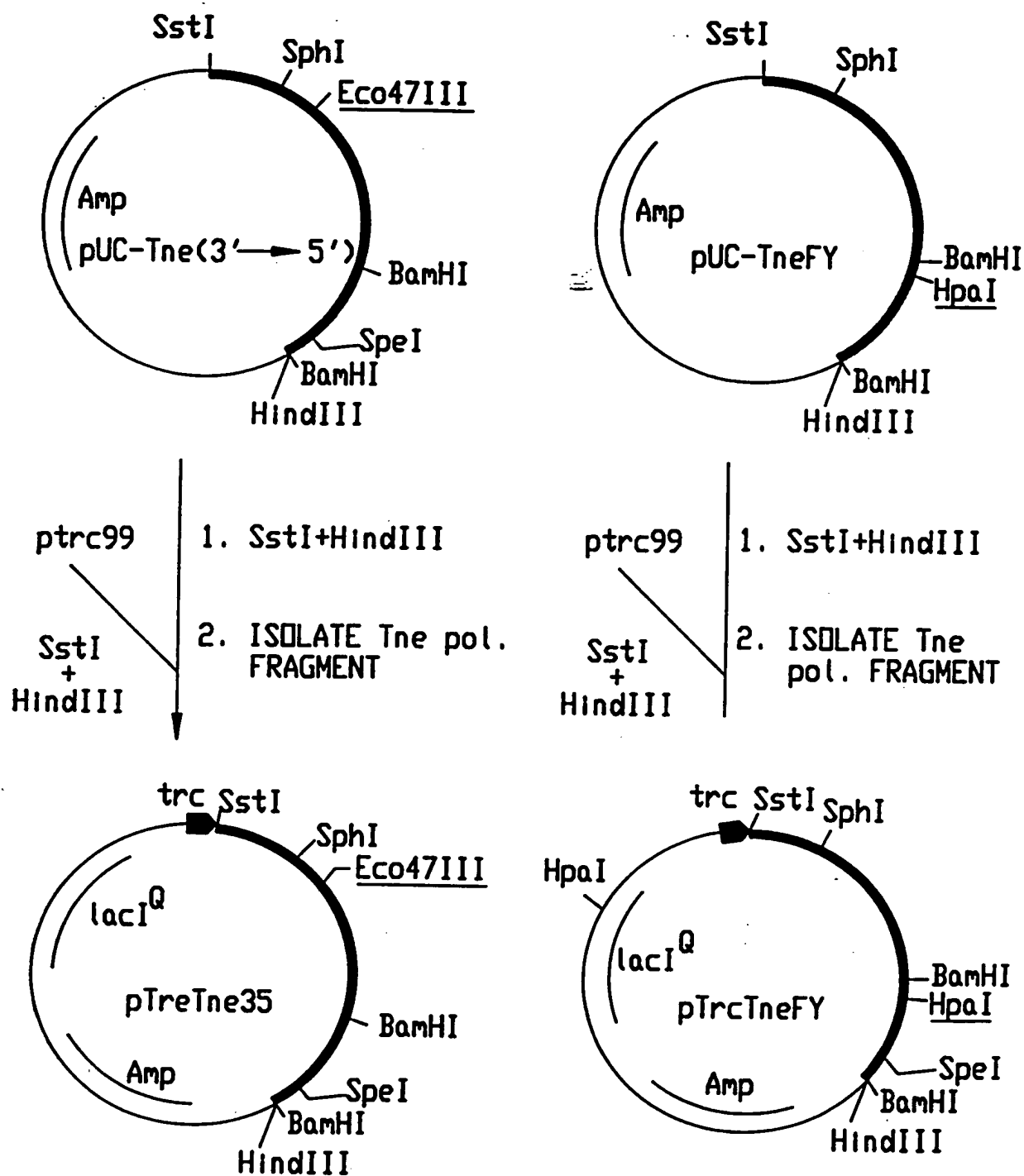


FIG.6B

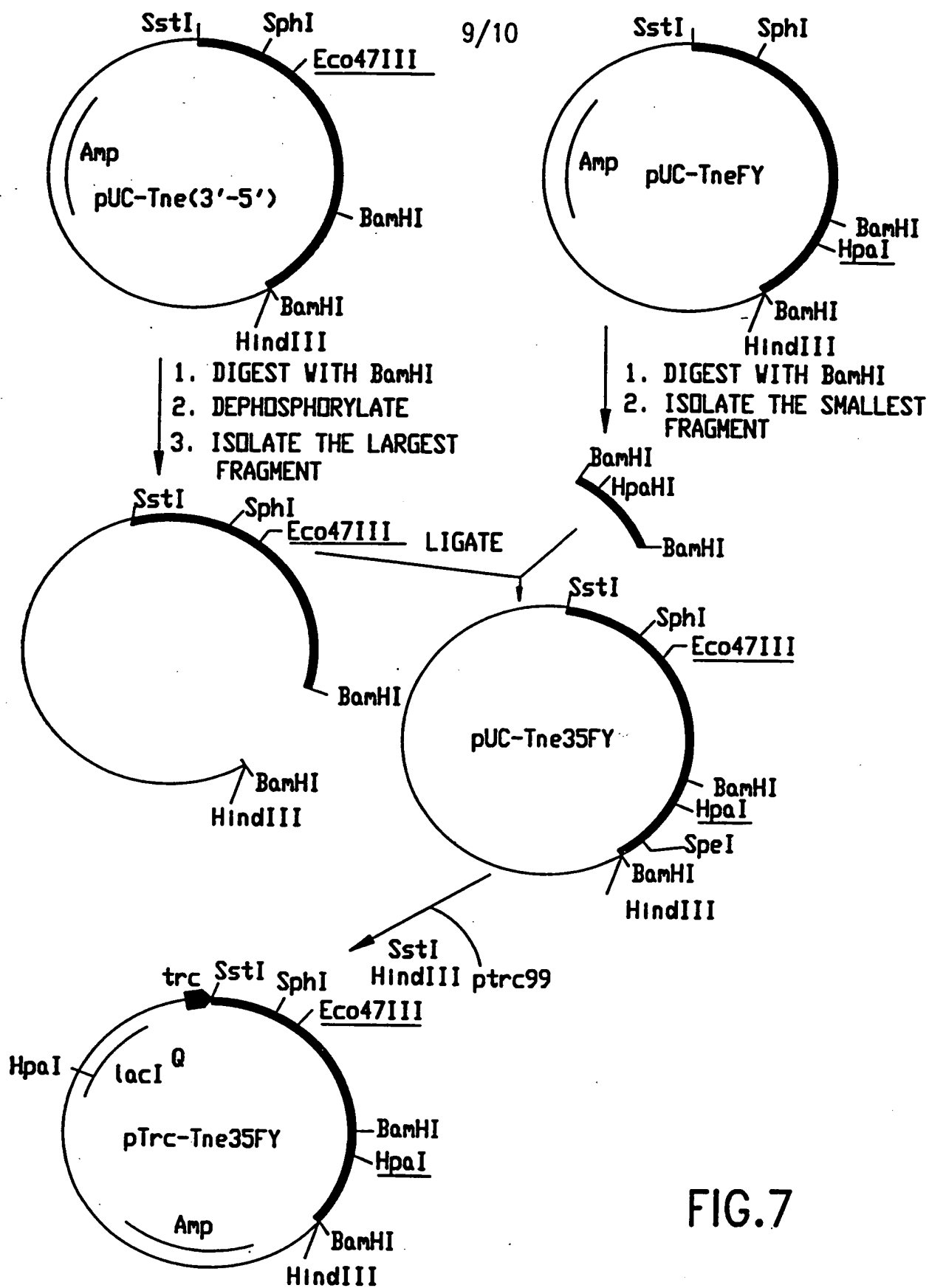


FIG.7

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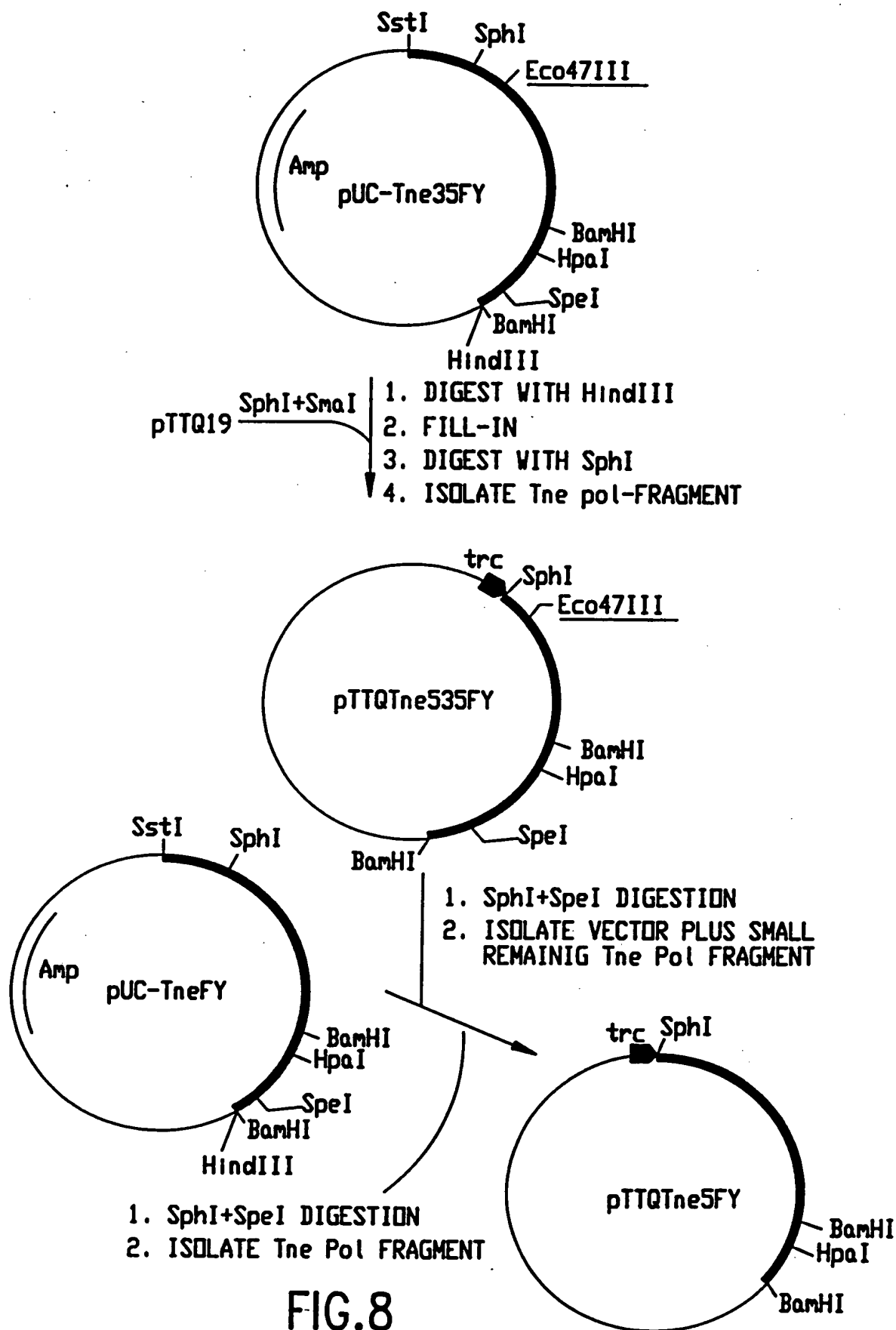


FIG.8

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/12358

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/54 C12N15/70 C12N9/12 C12N1/21 C12Q1/68
//(C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ARCHIVES OF MICROBIOLOGY, vol. 150, no. 1, 1988 SPRINGER, BERLIN, BRD, pages 103-104, H.W. JANNASCH ET AL. 'Thermotoga neapolitana sp. nov. of the extremely thermophilic, eubacterial genus Thermotoga' see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-70



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

28 February 1996

Date of mailing of the international search report

14.03.96

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Hornig, H

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/12358

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU-B- 8668891	28-04-92
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